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Stress Protein Synthesis in Human Keratinocytes Treated with Sodium Arsenite, Phenyldichloroarsine, and Nitrogen Mustard[1]

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Stress Protein Synthesis in Human Keratinocytes Treated with Sodium Arsenite, Phenyldichloroarsine, and Nitrogen Mustard. DEATON, M. A., BOWMAN, P. D., JONES, G. P., AND POWANDA, M. C. (1990). *Fundam. Appl. Toxicol.* **14,** 471–476. Cells from bacteria to man respond to sublethal thermal and certain chemical stresses by synthesis of heat shock, or stress, proteins. The human epidermal keratinocyte is a target for a variety of cytotoxic substances. One response of cells exposed to such agents may be the synthesis of stress proteins. Human epidermal keratinocytes were treated thermally (43°C) or chemically with sodium arsenite and the skin irritants phenyldichloroarsine and mechlorethamine. Proteins synthesized by keratinocytes were radiolabeled with [^{35}S]methionine, separated on polyacrylamide gels under denaturing conditions, and visualized by fluorography. Quantitation by computer-assisted densitometry of fluorograms revealed different patterns of synthesis of two heat shock proteins (hsp's) with apparent molecular weights of 70 and 90 kDa after treatment with heat, sodium arsenite, phenyldichloroarsine, or mechlorethamine. Sodium arsenite induced the highest levels of synthesis of these two proteins, approximately 10-fold and 3-fold increases in hsp-70 and hsp-90, respectively. Phenyldichloroarsine at 0.5 μM produced a 2-fold increase in hsp-70 but no significant increase in hsp-90. Mechlorethamine, in contrast, had an apparent inhibitory effect on hsp-70 synthesis. These results suggest that some but not all skin irritants induce the synthesis of heat shock proteins in human keratinocytes. © 1990 Society of Toxicology.

Exposure of cells to temperatures several degrees above those at which they normally grow causes the specific synthesis of several proteins and decreased synthesis of most others. This cellular reaction to temperature was originally termed the heat shock response, and the selected proteins that underwent increased synthesis were termed heat shock proteins, or hsp's. It is now known that a variety of agents other than heat, such as heavy metals, iodoacetamide (Levinson *et al.*, 1980), and ethanol (Li, 1983) can also induce this reaction, and there is a tendency now toward calling the response a stress, rather than heat shock, response (Schlesinger *et al.*, 1982).

Sodium arsenite induces the stress response in a variety of cells and organisms. Vossen *et al.* (1977) showed that Drosophila cells responded to sodium arsenite treatment with a chromosomal puffing pattern identical to that which occurred following heat shock. Levinson *et al.* (1980) and Johnston *et al.* (1980) demonstrated that sodium arsenite increased hsp synthesis in mammalian cells. Additionally, Brown and Rush (1984) demonstrated increased hsp synthesis in intact organs following intravenous administration of sodium arsenite. The exact mechanism by which heat or sodium arsenite induces the

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stress response is not known. Sodium arsenite is a potent sulfhydryl binding reagent (Fluharty and Sanadi, 1960), but it is not known whether this property is involved in the induction of the stress response.

Human keratinocyte cultures are a potentially valuable tool for testing cutaneous toxins. These cells are the principal target of such toxins, and they can be grown in serum-free medium (Boyce and Ham 1983). Thus, such cultures allow testing of the direct effect of substances, such as vesicants, on epidermal cells. Systemic complications of *in vivo* models makes them less suitable than *in vitro* models for studying mechanisms of action of cutaneous toxins.

To determine if the stress response might be activated in the response of human skin to vesicating agents such as phenyldichloroarsine (PDA), a Lewisite analog, or mechlorethamine (nitrogen mustand, NM), an analog of sulfur mustard, human epidermal keratinocytes were incubated with these at subcytotoxic concentrations and examined for the synthesis of hsp's.

We confirmed that hsp synthesis occurs in cultured keratinocytes after elevation of temperature as well as by sodium arsenite. While phenlydichloroarsine produced a small but significant increase in hsp synthesis, nitrogen mustard did not.

MATERIALS AND METHODS

Cell culture. Human epidermal keratinocytes and keratinocyte growth medium were obtained from Clonetics Corp. (San Diego, CA). The cells were passaged into tissue culture tubes (Ambitubes; 5.5 cm^2 growth area; Flow Laboratories, McLean, VA) or 96-well multiplates by removal from the original 25-cm^2 flask with 0.25% trypsin and 0.01% EDTA and subcultivated at a 1:10 split ratio.

Cytotoxicity. Cytotoxicity of test compounds was assessed by the method of Mosmann (1983). Briefly, keratinocytes were cultured to confluence in 96-well plates. One hundred microliters of fresh medium was added and the cells were treated with serial dilutions of sodium arsenite, PDA, or NM for 24 hr. At the end of this time, 20 μl of MTT (3-(4,5-dimethylthizol-2-yl)-2,5-diphenyltetrozolium bromide; Sigma, St. Louis, MO) was prepared by dissolving 5 mg/MTT/ml in phosphate-buffered saline, pH 7.5, and filtering through a 0.22-μm filter (Millipore, Bedford, MA); the MTT was then added to each well for 45 min at 37°C. At the end of this time, the media were removed by inverting the plates, and 100 μl of 1 N HCl-isopropanol (1:24 v/v) was added to extract and solubilize the formazan. The microplate was then read on a microplate reader (Vmax, Molecular Devices, Palo Alto, CA) at a 560-nm wavelength and data were presented as percentage of cells surviving calculated from amount of formazan per well relative to control.

Radiolabeling of cellular proteins. Upon achieving confluence the cultures were changed to fresh medium and cells were either heat-shocked or treated with various concentrations of sodium arsenite, PDA, or NM for 3 hr. Heat-shocked (HS) cultures were treated for 30 min at 43°C and then returned to 37°C for 3 hr. At the end of 3 hr, heat-shocked and chemically treated cultures were labeled for 1 hr in methionine-free medium containing 10 μCi/ml [^{35}S]methionine. At the end of the labeling period, radiolabeled medium was removed and the cell layer was rinsed three times with 4°C phosphate-buffered saline and once briefly with isotonic ethanol in water (1.34%) to remove residual salt. Cells were then lysed in 0.25 ml sample buffer containing 1% sodium dodecyl sulfate, 2% 2-mercaptoethanol, and 15% glycerol. Lysis was carried out until no intact cells could be seen by phase-contrast microscopy. The lysate was then heated to 95–100°C for 5 min to complete denaturation of samples, followed by centrifugation at 11,500*g* for 30 min to rid the samples of particulates.

To determine the amount of [^{35}S]methionine incorporated into protein, a 5-μl aliquot was drawn from each sample and precipitated by adding an equal volume of ice-cold 10% trichloroacetic acid (TCA). Each precipitate was collected onto a 25-mm-diameter, 0.45-μm filter, dried, and placed in 20 ml of scintillation fluid. Total counts per minute (cpm) of the 5-μl sample were determined by scintillation counting. Using these counts, sample volumes were adjusted during loading of the samples onto polyacrylamide gels such that each lane on any given gel contained an equal amount of TCA-precipitable radioactivity. For a given gel, the sample with the least amount of radioactivity determined the amount of radioactivity that was applied for all other lanes.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out according to the method of Laemmli (1970). Stacking gels were 2 cm long and consisted of 4% acrylamide buffered to pH 6.8. The running gels were 12 cm long, 10% acrylamide, and pH 8.8. Electrophoresis was carried out at 35 mA per gel with cooling.

After electrophoresis, proteins in the gels were stained with Coomassie brilliant blue R-250. Radiolabeled proteins in the gels were made visible on X-ray film using the fluorography technique of Jen and Thach (1982). Stained gels and fluorograms were scanned with a laser densitometer (LKB Ultroscan XL, Bromma, Sweden) to quanti-

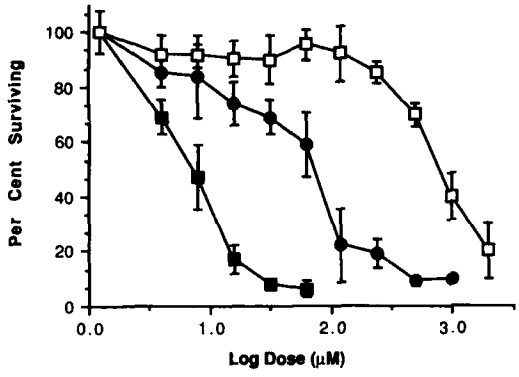

FIG. 1. Cytotoxicity of sodium arsenite (—□—), nitrogen mustard (—●—), and phenyldichloroarsine (—■—) to human epidermal keratinocytes as assessed by the MMT/formazan method. Percent Surviving represents the percentage of formazan product per well relative to control after a 24-hr treatment. Each point represents the results of quadruplicate measurements. Error bars are standard deviations as a percentage.

tate the amounts (in stained gels) and levels of synthesis (fluorograms) of stress proteins present. The output of the densitometer was verified from densitometry calibration standards.

Data analysis. The data were first subjected to one-way analysis of variance. If a significant difference occurred, then Dunnett's *t* test was used to determine which means differed from control. A significance level of 0.05 was used for all statistical tests (Gad and Weil, 1989).

RESULTS

Figure 1 illustrates the cytotoxicity of sodium arsenite, nitrogen mustard, and phenyldichloroarsine on human epidermal keratinocytes. Cytotoxicity indicated by observation under phase contract microscopy of rounded up cells (cells having decreased adherence) did not become apparent until 6 hr of incubation and then only at the highest concentration tested for each compound. By 24 hr, however, considerable cell loss occurred at the higher concentrations of each compound and no formazan reaction product was detectable. It is apparent that phenyldichloroarsine was the most cytotoxic followed by nitrogen mustard and sodium arsenite. The concentrations of test compounds used for assessment of stress response were based on amounts that showed low cytoxicity and no cell loss during a 24-hr incubation with test substance.

Fluorograms of proteins separated by SDS–PAGE revealed that elevating the temperature of keratinocyte cultures for 30 min caused two proteins with molecular weights (MW) of 70 and 90 kDa to undergo increased synthesis (Fig. 2). By convention, these are referred to as hsp-70 and hsp-90. In control keratinocyte cultures hsp-70 was responsible for about 2.2% of the total radiolabel resolved on the fluorograms, while hsp-90 was responsible for approximately 2.8% of the radiolabel. Following heat shock, however, hsp-70 and hsp-90 accounted for 7.9 and 5% of the radiolabel, respectively (Fig. 3).

Of the three compounds tested, sodium arsenite had the greatest effect on stress protein production in the cultured keratinocytes. At the optimal concentration of 50 μM, sodium arsenite stimulated an approximate 10-fold increase in hsp-70, and hsp-90 was increased almost 3-fold. At 250 μM, sodium arsenite may be approaching a dose which is inhibiting cellular metabolism so that the cell is unable to respond.

Although not as effective as sodium arsenite, the vesicant PDA also induced a significant increase in the production of hsp-70. An approximate twofold increase occurred at a concentration of 0.5 mM PDA. Hsp-90, however, was not elevated significantly above control.

In contrast to sodium arsenite and PDA, nitrogen mustard had an inhibitory effect on hsp-70 production at concentrations determined nontoxic to the cells. Hsp-90 was unchanged at the lower two concentrations and inhibited at the highest concentration tested, 50 μM.

DISCUSSION

The heat shock, or stress, response is operationally defined by enhanced incorporation

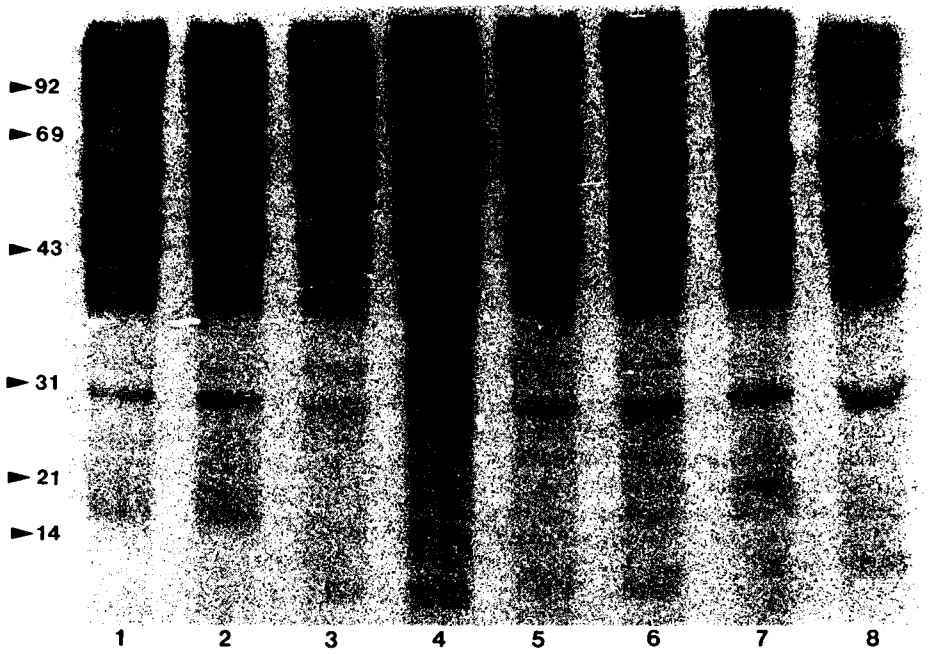

FIG. 2. A fluorogram of an SDS–polyacrylamide gel comparing human epidermal keratinocyte cultures treated with various inducers of the stress response. Lane 1, no treatment; lane 2, heat shock; lane 3, 50 μM sodium arsenite; lane 4, 100 μM sodium arsenite; lane 5, 250 μM sodium arsenite; lane 6, 0.5 μM PDA; lane 7, 1.0 μM NM; lane 8, 10 μM NM.

of a radiolabeled amino acid into a few cellular proteins termed heat shock proteins. It appears that synthesis of the remaining proteins, which number approximately 2000 by two-dimensional gel electrophoresis (Garrels 1989), is suppressed during this time, implying that a significant portion of the cells' energy is being diverted to this effort. An apparently basic response to environmental challenges, the stress response has been observed following exposure to heat, toxic chemicals, and radiation. (See review by Nover, 1984.) Of course, although a cell must be damaged in some way for the induction of stress proteins to occur, the cell's protein synthesizing machinery must still be intact. Observed in all cells examined from bacteria to man (Schlesinger *et al.*, 1982), in general, its induction seems to confer a protective effect on cells to subsequent insult (Subjeck, *et al.*, 1982; Lindquist, 1986). The specific functions of the stress proteins are not known, but it has been suggested that they help solubilize and refold denatured proteins (Deshaies *et al.*, 1988).

In the present study, it was hypothesized that skin keratinocytes would be induced to manufacture stress proteins following exposure to heat or toxic substances. The results indicate that both heat shock and sodium arsenite induced the full stress response in human epidermal keratinocytes as indicated by increased synthesis of both hsp-70 and hsp-90. These results agree well with results that have been obtained with peripheral lymphocytes (Eid *et al.*, 1987) and human fibroblasts (Levinson *et al.*, 1980). PDA was only mildly stimulatory, however, and NM appeared to inhibit induction.

The two proteins hsp-70 and hsp-90 did not respond in synchrony to the conditions used. When the cells were stressed with heat or sodium arsenite, hsp-70 underwent a much greater change in synthesis than hsp-

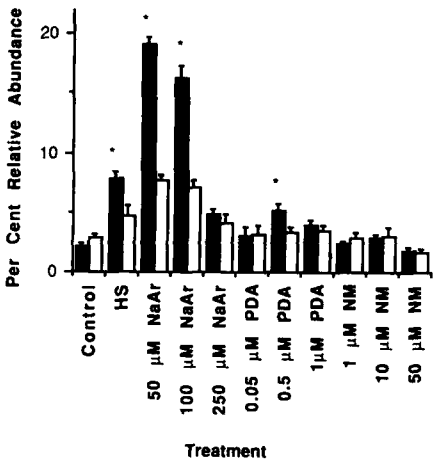

FIG. 3. Summary of hsp-70 and hsp-90 synthesis by normal human epidermal keratinocytes after various treatments. Percent Relative Abundance is the percentage of the total resolved radiolabel attributable to hsp-70 or hsp-90. Error bars are standard error of the mean. Data from three to seven gels were pooled to give mean of relative abundance. Asterisks indicate a significant difference from control at the 0.05 level by Dunnett's *t* test.

90. This was also true to some extent of the response to PDA. These asynchronous changes suggest that while both hsp-70 and hsp-90 may be involved in the stress response, their syntheses may be regulated by different mechanisms. It is not known why induction of stress proteins in response to 250 μM sodium arsenite was less than the response to 100 μM. Although neither was cytotoxic on the basis of the MMT viability assay, perhaps protein synthesis was compromised enough at 250 μM to inhibit the stress response.

Because it is a target for a variety of irritating substances that produce inflammation, the human epidermal keratinocyte was chosen as a model system for this study. The vesicant PDA, an organic arsenical, is probably able to penetrate the skin more readily than arsenite and this may explain its high potency as a vesicant and relatively high potency as a cytotoxic agent. Sodium arsenite and PDA are both sulfhydryl binding agents (Fluharty and Sanadi, 1960; Dill *et al.*, 1987), and it has been proposed that a sulfhydryl containing target is essential for activation of the stress response (Levinson *et al.*, 1980), but further study is needed to establish a correlation between this property and induction of stress proteins in keratinocytes. Nitrogen mustard is a mild vesicant. Its ability to damage DNA may prevent activation of gene expression, thereby preventing the stress response. Anathan *et al.* (1986) have proposed that the common denominator in the stimulation of this response is denaturation of protein. Nitrogen mustard may not have a denaturing effect on proteins, further explaining its failure to induce the stress response. Although this study does not provide evidence for mechanisms of actions of the toxins tested, the differential levels of hsp production in the cells suggest that the toxins affect skin via different mechanisms. It also indicates that not all vesicants induce synthesis of stress proteins.

Efforts to use cultured cells for testing potentially toxic substances have generated numerous *in vitro* tests which rely on the measurement of irreversible cellular damage (Cook and Mitchell, 1989). Although it appears that not all toxins elicit the stress response, for certain types of compounds its induction might represent a more sensitive test—predictive at noncytotoxic doses—than is currently available.

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